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NOX3-derived reactive oxygen species promote TNF-α-induced reductions in hepatocyte glycogen levels via a JNK pathway

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ABSTRACT

TNF-α-induced insulin resistance is associated with generation of reactive oxygen species (ROS). This study aims at defining the link between ROS production and hepatic insulin resistance. Treatment with TNF-α increased ROS generation through activating NADPH oxidase 3 (NOX3) in HepG2 hepatocytes. Down-regulation of NOX3 using siRNA prevented TNF-α-induced decrease of cellular glycogen. In the cells treated with TNF-α, there were NOX3-dependent activation of JNK, inhibition of IRS1 and phosphorylation of AKT/PKB and GSK. In conclusion, the effects of TNF-α on hepatic insulin resistance appear to be, at least in part, mediated by NOX3-derived ROS through a JNK pathway.

1. Introduction

Insulin resistance, defined as a diminished ability of cells such as adipocytes, skeletal muscle cells and hepatocytes to respond to the action of insulin, is a key feature of type 2 diabetes. In hepatocytes, insulin resistance is brought about by a combination of pathological alterations: hyperglycaemia, hyperinsulinaemia, oxidative stress and altered profiles of adipocytokines.

A large body of evidence shows that oxidative stress, generated through increased generation of reactive oxygen species (ROS), can be observed in insulin resistant cells and tissues [1]. The enhanced ROS production is thought to lead to abnormal intracellular signaling and thereby causally contribute to the development insulin resistance [2]. Decreased glycogen levels are a hallmark of insulin-resistant hepatocytes; underlying mechanisms include decreased glycogen synthesis and failure to suppress glucose production [3].

TNF-α has been recognized as an important mediator of insulin resistance by impairing insulin signaling [4]. Moreover, it has been well demonstrated that TNF-α may lead to increased ROS levels, which may induce various signaling pathways including p38MAPK, ERK and JNK activation. However, little is known about the cellular mechanisms of ROS generation in TNF-α-induced hepatic insulin resistance and the link between ROS and hepatic insulin resistance.

ROS are produced via multiple processes such as mitochondrial electron transport chain, nitric oxide synthase, xanthine oxidase, as well as NOX family NADPH oxidases [5]. Recent attention has been focused on NOX enzymes as a potential source of ROS production in insulin resistance [6]. The NOX family consists of several members, with distinct cellular and subcellular distribution, as well as distinct cellular functions [7]. A role for NOX3 in insulin action in hepatocytes has been suggested [8].

Here, we test the hypothesis that NOX3-derived ROS mediate the TNF-α effect on hepatocytes, particularly the drop in cellular glycogen content. We provide evidence that the effects of TNF-α appear to be, at least in part, mediated by NOX3-derived ROS through a JNK pathway.

2. Materials and methods

2.1. Analysis of cellular glycogen content

Glycogen levels were measured in cells incubated for 3 h in the presence of 1 nmol/l insulin (Usbio) using a glycogen assay kit (Biovision).

Abbreviations: DPI, diphenyliodinium; IRS, insulin receptor substrate; JNK, Jun N-terminal kinase; L-NAME, N^G-nitro-L-arginine methyl ester; NOX, NADPH oxidase; PKC, protein kinase C; RNAi, RNA interference; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α

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2.2. Determination of ROS

Cells (3×10^5 cells/ml) were incubated with 5 μmol/l of DCF-DA (Sigma) for 40 min at 37 °C. The DCF fluorescence was measured by FACS.

The sections of OCT-embedded liver tissues were incubated with 10 μmol/l DHE (Sigma) for 15 min at room temperature. The sections were analyzed by fluorescence microscopy.

2.3. RNA extraction, RT-PCR and real-time PCR

Extraction of total RNA (Trizol, Invitrogen), RT (A3500 kit, Promega), and real time PCR (7500 system, ABI) were performed according to manufacturer's instruction.

2.4. Immunofluorescence and immunohistochemistry

The coverslips of HepG2 or sections of OCT-embedded liver tissues of rats were incubated with polyclonal antibodies at 37 °C for 60 min then labeled with TRITC-conjugated anti-rabbit IgG at 37 °C for 60 min. Finally, the coverslips were mounted with DABCO.

2.5. siRNA transfection

The siRNA targeting NOX3 mRNA were transfected into HepG2 using Tran Messenger TM Transfection Reagent (Qiagen) according to manufacture's instruction.

2.6. Western blot

Cell lysates (30–60 μg protein) were separated by 10% SDS–PAGE, transferred to PVDF membrane (Millipore), blocked with 5% non-fat dry milk, and probed with antibodies at 4 °C overnight. The blots were incubated with HRP-conjugated anti-IgG, followed by detection with ECL (Santa Cruz).

2.7. Statistical analysis

All values are represented as means ± S.E.M. of the indicated number of measurements. One-way ANOVA test was used to determine significance, requiring $P < 0.05$ for statistical significance.

3. Results and discussions

TNF-α has been implicated in the pathogenesis of insulin resistance in vitro and in vivo [9]. In the first set of experiments, we fed rats a high-fat diet to induce insulin resistance. After 12 weeks, the rats weighed 20% more than control rats, but still maintained normal blood glucose levels (data not shown). However, plasma insulin levels in the rats fed with high-fat diet were increased, demonstrating decreased insulin sensitivity (Fig. 1A). Rats under high-fat diet showed elevated plasma TNF-α level (Fig. 1B). Hepatic glycogen levels in rats fed with high-fat diet were significantly decreased, confirming insulin resistance (Fig. 1C). ROS have been proposed to play a causal role in insulin resistance [10]. ROS measurement in hepatic tissue slices showed enhanced ROS production in rats fed a high-fat diet (Fig. 1D). These observations raised the possibility that ROS may be the link between TNF-α and insulin resistance.

We next sought to extend these observations from an in vivo model to a cellular model of insulin resistance. In cultured HepG2 cells, TNF-α has been suggested to induce insulin-resistance, as assessed by their decreased capacity to accumulate glycogen in the presence of insulin. To exclude the side effects associated to TNF-α, such as apoptosis, we quantified the cell viability in TNF-α-treated HepG2 by MTT assay. The results indicated no cytotoxicity in connection with the time of exposure to TNF-α (4 ng/ml, 2–4 days) (Fig. 2A) and the dose of TNF-α (2–6 ng/ml, 4 days) (Fig. 2B) in HepG2. Indeed, TNF-α (4–6 ng/ml, 4 days) led to a dose-dependent decreased glycogen content of HepG2 (Fig. 2C). We then investigated whether ROS levels were altered by TNF-α treatment. As shown in Fig. 2D, ROS levels were dose-dependently increased by exposure of cells to TNF-α. To identify the source of ROS generated in response to TNF-α, we studied the effects of different inhibitors of ROS-generating systems: DPI (NOX NADPH oxidase, 5 μmol/l), L-NAME (nitric oxide synthases, 100 μmol/l), rotenone (mitochondrial respiratory chain, 2 μmol/l) and oxypurinol (xanthine oxidase, 100 μmol/l). Similarly, there was no cytotoxicity in inhibitors-treated HepG2 (Fig. 2E). However, only DPI inhibited the generation of ROS in response to TNF-α (4 ng/ml, 4 days) (Fig. 2F), raising the possibility that a NOX enzyme could be the source of ROS.

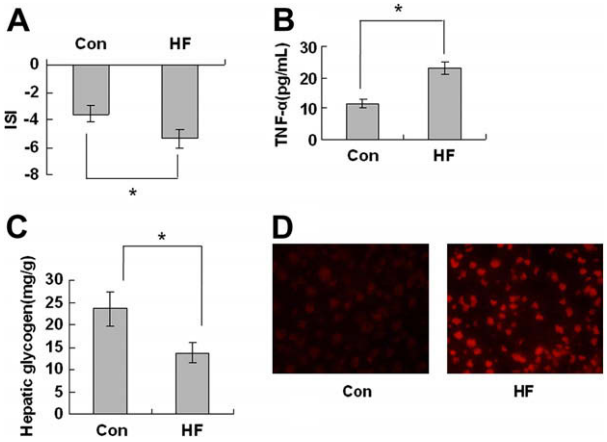

Fig. 1. Analysis of insulin sensitivity, plasma TNF-α, intracellular glycogen, and ROS in rats on high-fat diet. Rats were on a high fat diet and insulin sensitivity index (ISI) (ln[1/(fasting blood glucose × fasting serum insulin)]) (A), plasma TNF-α levels (B), liver intracellular glycogen content (C), and liver tissue ROS generation assessed by DHE fluorescence (D) were determined. Data are presented as mean ± S.E.M., $n = 6$. $^*P < 0.05$ by ANOVA test.

We therefore analyzed the expression of NOX isoforms and subunits in non-treated and TNF-α-treated HepG2. Using RT-PCR, we found expression of NOX3, but not NOX1, NOX2, NOX4 and NOX5 in non-treated and TNF-α-treated HepG2, indicating that TNF-α-induced ROS might be derived from NOX3 (Fig. 2G). We also found p22phox, p67phox, p47phox and Rac1 (Fig. 2G). A similar expression pattern of NOXs and partners was found in hepatic tissues (data not shown). Previous studies have demonstrated the absence of NOXO1 and NOXA1 in HepG2 [8], raising the possibility that p47phox might function as an activator subunit for NOX3 in HepG2.

We next examined the effect of TNF-α treatment on expression and activity of NOX3 in HepG2. Interestingly, TNF-α up-regulated the expression of NOX3, but not p22phox, p47phox, p67phox and Rac1 as shown in Fig. 3A. Importantly, the expression of NOX3 was not only enhanced in response to TNF-α in vitro, but also in response to high fat diet in vivo (Fig. 3B). Protein kinase C (PKC) regulates the activation NOX2, and may activate NOX3, at least with p47phox as organizer subunit [11]. We therefore investigated the role of PKC in TNF-α-induced ROS generation. PMA induced a strong ROS generation, which was not enhanced by the addition of TNF-α, suggesting that they act within the same pathway. The PKC inhibitor hypericin (4 μmol/l, preincubation for 30 min) inhibited TNF-α-induced ROS generation (Fig. 3D) but without cytotoxicity (Fig. 3C). From the two known NOX activator subunits, only p47phox, but not NOXO1 is expressed in HepG2 cells, raising the

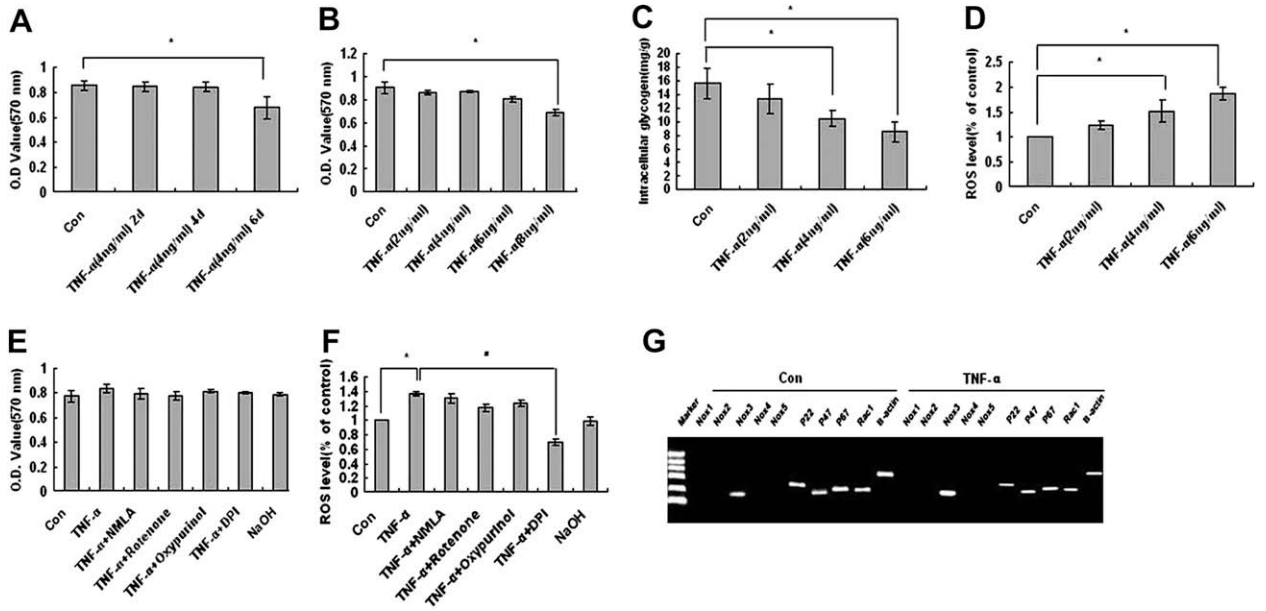

Fig. 2. TNF-α-induced increased ROS generation through expression of NOX enzymes. No cytotoxicity in connection with the time of exposure to TNF-α (4 ng/ml, 2–4 days) (A), and the dose of TNF-α (2–6 ng/ml,4 days) (B), in HepG2 cells as indicated by MTT assay. TNF-α (4–6 ng/ml, 4 days) decreased intracellular glycogen levels (C), and stimulated ROS production detected by DCF (D), in a concentration-dependent manner. No cytotoxicity in inhibitors-treated HepG2 (E). DPI impaired TNF-α-induced ROS generation (F). The mRNA expression profile of NOX isoforms and subunits in non-treated and TNF-α-treated HepG2 (G). Data are presented as mean ± S.E.M., $n = 3$ independent experiments. $^{*}P$ and $^{\#}P < 0.05$ by ANOVA test.

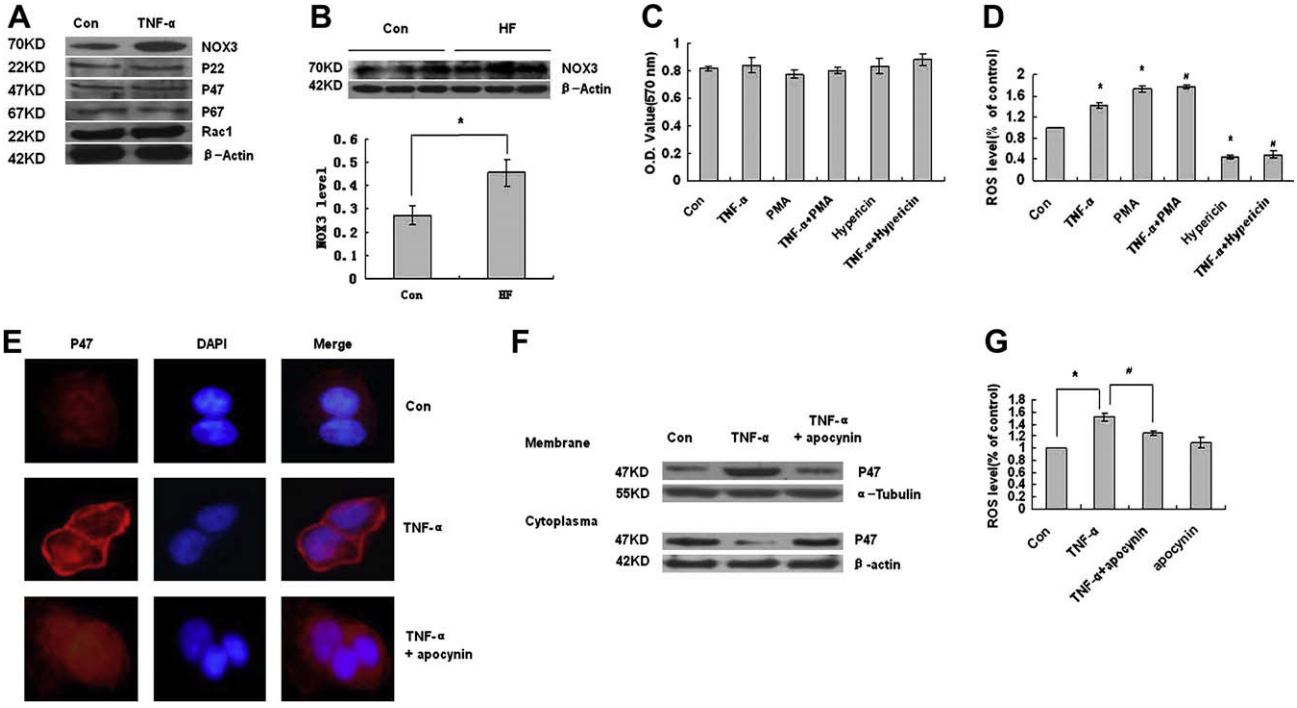

Fig. 3. TNF-α-induced enhanced expression and activity of NOX3. Expression of NOX3 in HepG2 treated with 4 ng/ml TNF-α for 4 days (A) and livers of rats fed a high-fat diet for 12 weeks (B). Effect of the PKC activator PMA (1 μmol/l) and the PKC inhibitor hypericin (4 μmol/l) on cell viability (C) and TNF-α-induced ROS generation in HepG2 (D). Subcellular localization of p47phox in TNF-α-treated HepG2 cells, as measured by confocal immunofluorescence microscopy and western blot (E and F). Impact of apocynin on TNF-α-induced ROS generation (G). Data are presented as mean ± S.E.M., $n = 3$ independent experiments. $^{*}P < 0.05$ compared with control and $^{\#}P < 0.05$ compared with TNF-α treatment by ANOVA test.

possibility that translocation of p47phox plays a role in the activation of NOX3. In unstimulated cells, p47phox exist in the cytosol, while upon stimulation it translocates to the plasma membrane [12]. We have therefore investigated subcellular localization of p47phox by confocal immunofluorescence microscopy. As shown in Fig. 3E, p47phox had a cytoplasmic distribution in unstimulated HepG2 cells, but showed a plasma membrane distribution in response to TNF-α stimulation. Furthermore, membrane proteins were extracted to confirm TNF-α-induced membrane distribution of p47phox (Fig. 3F). The antioxidant/NOX inhibitor apocycin has

been suggested to act, in least in part, through inhibition of p47phox translocation [5]. Fig. 3E and F demonstrate that apocynin (100 μmol/l, 30 min) prevented TNF-α-induced p47phox translocation. Apocynin also prevented the increase in ROS levels in response to TNF-α (Fig. 3G). Taken together, the results demonstrate that TNF-α enhanced the production of ROS through two mechanisms: (i) increased NOX3 protein levels, and (ii) PKC-dependent NOX3 activation, presumably involving p47phox translocation.

To further explore the role of NOX3 in TNF-α-induced increased ROS generation and decreased glycogen level in hepatocytes, we transiently transfected siRNA targeting NOX3 (siRNA–NOX3) into HepG2. Analysis by real-time PCR indicated that the amount of NOX3 mRNA was markedly down-regulated both in control and in TNF-α-treated cells, while control siRNA had no effect (Fig. 4A). Similarly, NOX3 protein was reduced following siRNA–NOX3 transfection both in control and TNF-α-treated cells (Fig. 4B). NOX3 siRNA led to an approximately 50% decrease of basal ROS generation, but completely abolished TNF-α-induced ROS generation (Fig. 4C). Thus, while several sources of ROS might participate in basal ROS generation in HepG2 cells, it appears that NOX3 is the predominant source of ROS induced by TNF-α. We next investigated the role of NOX3 in the regulation of intracellular glycogen levels (Fig. 4D). NOX3 siRNA did not impact glycogen levels under control conditions, but completely prevented the TNF-α-induced decrease of glycogen. These data demonstrate that NOX3-derived ROS are essential mediators of the TNF-α-induced decreased glycogen levels in hepatocytes.

Finally, we investigated downstream pathways translating elevated ROS levels into decreased glycogen levels. One attractive possibility is that ROS-induced insulin resistance is mediated by JNK. ROS promote activation of JNK [13] and JNK deletion or inhibition improves insulin sensitivity in mice [14,15]. Our results show that JNK was activated in response to TNF-α, and that effect was reversed by NOX3 down-regulation (Fig. 5A). In parallel with increased phosphorylation of JNK, TNF-α down-regulated levels of IRS1 and stimulated its inhibitory phosphorylation on Ser307 (Fig. 5B). This corroborates previous studies [16]. Importantly, the decreased IRS1 protein levels and the increased Ser307 phosphorylation were reversed by NOX3 siRNA, to a similar extend as observed with SP600125 (Fig. 5B). Moreover, pretreatment with SP600125 (JNK inhibitors) can reverse the decrease of cellular glycogen levels induced by TNF-α (Fig. 5C). The TNF-α-induced impaired phosphorylation of AKT and GSK was rescued by siRNA-mediated NOX3 reduction (Fig. 5D and E). Thus, NOX3-derived ROS play a key role in TNF-α signaling towards insulin resistance.

In conclusion, our data indicate a key role of NOX3-derived ROS in TNF-α-induced insulin resistance in hepatocytes: (i) NOX3

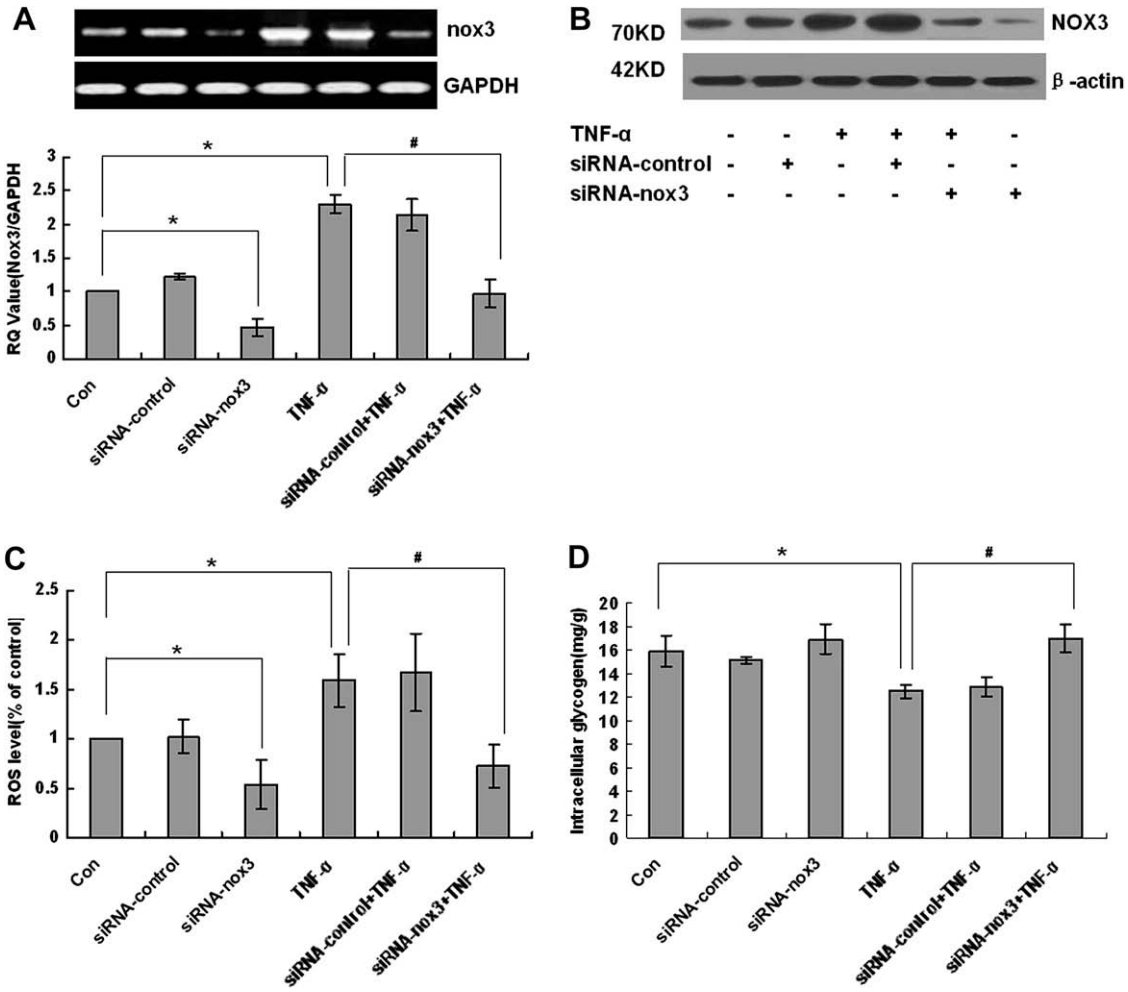

Fig. 4. Effects of NOX3 siRNA on TNF-α-induced alterations of ROS generation and cellular glycogen levels. HepG2 were transiently transfected with siRNA–NOX3 and analyzed after 4 days. When indicated, cells were incubated on day 3 and 4 with TNF-α (4 ng/ml). NOX3 mRNA levels detected by real-time PCR (A); NOX3 protein levels (B); cellular ROS generation (C), and cellular glycogen levels (D). Data are presented as mean ± S.E.M., $n = 3$ independent experiments. $^{*}P$ and $^{\#}P < 0.05$ by ANOVA test.

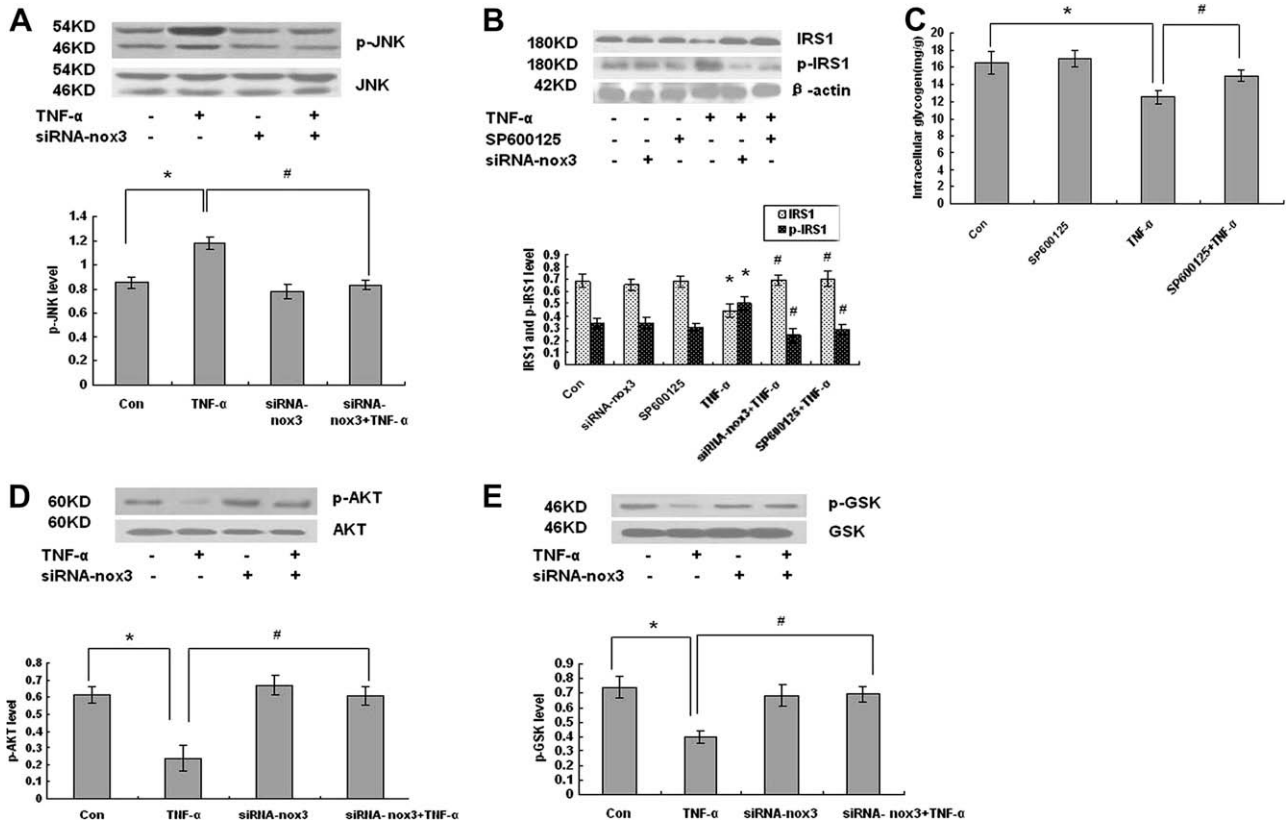

Fig. 5. Influences of NOX3 siRNA on TNF-α-induced alterations of signaling pathways. HepG2 were transiently transfected with siRNA–NOX3 and analyzed after 72 h. When indicated, TNF-α (4 ng/ml) and/or the JNK inhibitor SP600125 (20 μmol/l) was included after 48 h. Western blots of total and phosphorylated protein are shown for JNK (A), IRS1 (B), AKT/PKB (D), and GSK (E). The anti-phosphoIRS1 antibody recognized Ser307. Pretreatment with SP600125 for 30 min can reverse the decrease of cellular glycogen levels induced by TNF-α (4 ng/ml, 48 h) (C). All experiments were performed in the presence of 1 nmol/l insulin. Data are presented as mean ± S.E.M., $n = 3$ independent experiments. $^{*}P < 0.05$ compared with control and $^{\#}P < 0.05$ compared with TNF-α treatment by ANOVA test.

is the predominant source of TNF-α-induced ROS generation, and (ii) this ROS generation is critically involved in the alterations of intracellular signaling associated with insulin resistance and in the resulting decrease of intracellular glycogen levels.

The association of NOX3 and insulin resistance is not only observed in the HepG2 cell line, but also observed in the liver of rats fed with high fat diet. However, the relative importance of NOX3 as source of ROS generating insulin resistance in vivo needs further investigation. Also, the upregulation and activation of NOX3 by TNF-α is of interest. In particular, our results suggest that PKC activation and p47phox translocation might activate NOX3 in hepatocytes, while in the inner ear, NOX3 appears to be associated with NOXO1 as an activator subunit [11].

Redox activation of JNK is well known and likely to play a role in the NOX3-dependent induction of insulin resistance: JNK induces inhibitory serine-phosphorylation and degradation of IRS-1, leading to diminished PI3-kinase activity and a subsequent drop in AKT/PKB and GSK phosphorylation, finally resulting in decreased glycogen synthesis [17]. However, further studies are needed to determine whether NOX3 acts exclusively through this pathway or whether additional signaling steps might be involved.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.01.044.

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